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THE INFLUENCE OF NITROXYL RADICAL 4-TRIPHENYLPHOSPHONIOACETAMIDO-2,2,6,6-TETRAMETHYLPIPERIDINE-1-OXYL (TPPA-TEMPO) ON GENERAL TOXICITY AND THERAPEUTIC EFFICIENCY OF CYCLOPHOSPHAMIDE ON TRANSPLANTABLE MOUSE LYMPHOSARCOMA LS IN VITRO AND IN VIVO

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ABSTRACT

Systemic administration of nitroxyl radical 4-triphenylphosphonioacetamido-2,2,6,6-tetramethylpiperidine-1-oxyl chloride (TPPA-TEMPO) in subtoxic doses produces moderate inhibitory effect on the growth of mouse lymphosarcoma LS, but does not prolong the lifespan of animals. The latter was due to the light toxic effect of TPPA-TEMPO on animal's visceral organs, especially heart, lungs and kidneys. The administration of TPPA-TEMPO in conjunction with cyclophosphamide (CP) increases anti-tumor therapeutic efficacy which is expressed in both the increase in the life span of tumor bearing animals and in the number of mice cured. However, the values of both effects were not equally dependent on the duration of drug application, presumably, due to the side effects of TPPA-TEMPO on the organisms of the animals. The in vitro studies in the tumor cells cultures have shown that TPPA-TEMPO in small doses decrease metabolism and proliferation rate of tumor cells without affecting cell viability. It was assumed that antitumor effect of TPPA-TEMPO can be associated with either its direct impact on metabolism of tumor cells or the increase in the activity of phagocytes which take part in the mechanisms of antitumor resistance.

KEYWORDS: Nitroxide, Antioxidant, Cyclophosphamide (CP), Mice, Lymphosarcoma LS, Antitumor Activity, in Vitro, in Vivo, Therapeutic Efficacy

INTRODUCTION

Tissue injury by reactive oxygen species (ROS) formed in mitochondria both in normal redox reactions and under oxidative stress can be a reason for the development and progression of various diseases including malignant tumors. Antioxidants reacting with active forms of oxygen are able to decrease their pathogenic activity and contribute to antitumor resistance. This has been repeatedly shown for such well-known antioxidants as
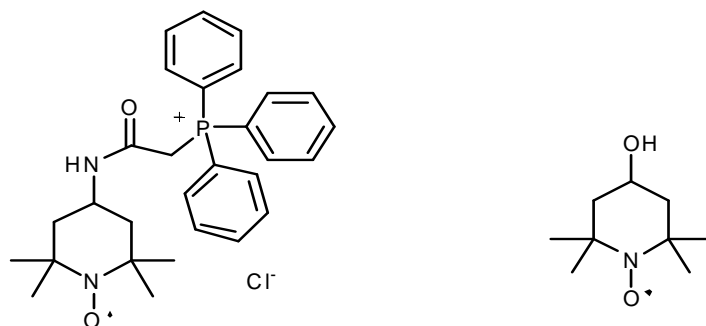
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vitamins E and C, carotenoids, etc.[1]. Over the recent years, a lot of data on antioxidant properties of nitroxides and their therapeutic effects have been published [2-7].

Usually, biological activity of nitroxides is associated with their antioxidant properties [1]. However, it has been shown that apart from general antioxidant effects they reveal various individual biological effects [3,7] varying from cito- and genototoxic [8] ones to the effects inducing the activity of some cellular enzymes. Supplementation with nitroxides may improve the results of antitumor drug therapy (e.g. doxorubicine, cyclophosphamide) and may enhance the effect of hyperthermia by induction of tumor cells apoptosis [6-12].

In the work [13] we have studied the effect of nitroxide Tempol and its conjunctions with cytostatic drug cyclophosphamide (CP) on the growth of transplantable lymphosarcoma LS in mice. This tumor is highly sensitive to apoptosis induction. We have demonstrated that the therapeutic effect of CP decreases rather than increases after Tempol supplementation. It has been found out that Tempol stimulates the activity of aldehyde dehydrogenase (ADG), an enzyme that greatly contributes to CP inactivation decreasing the yield of the main active metabolite of CP – phosphoramide mustard which is responsible for antitumor effect of CP. The effect of Tempol-induced ADG gene expression on the given model by far exceeds the antioxidant effect of the nitroxide.

Mitochondria targeting may amplify the effect of antioxidants because they are accumulated exactly at the place where major part of cellular ROS is generated [14]. In this work we studied the activity of another nitroxide - 4-triphenylphosphonioacetamido-2,2,6,6-tetramethylpiperidine-1-oxyl chloride (TPPA-TEMPO) (synthesis and preliminary results on the efficacy of antitumor therapy have been reported in [15]). This nitroxide contains a triphenylphosphonium group, which causes intramitochondrial accumulation driven with transmembrane electrostatic potential and has revealed high antioxidant activity [15].

Recently a pioneering view on the mechanisms of anticancer activity of mitochondria-targeted antioxidants has been published [16]. It has been shown that scavenging of mitochondrial superoxide with TPPA-TEMPO (mitoTEMPO) inhibits cell growth, reduces viability, and induces apoptosis in melanoma cells but does not affect nonmalignant skin fibroblasts. We have extended the study of antitumor activity of TPPA-TEMPO and investigated the mechanisms involved. In particular, the influence of TPPA-TEMPO on the development of animals immune response to the transplanted tumor in animals and its direct effects on the tumor cells and macrophages have been studied both *in vivo* and *in vitro*.

TPPA-TEMPO   Tempol

Figure 1

The Influence of Nitroxyl Radical 4-Triphenylphosphonioacetamido-2,2,6,6-
Tetramethylpiperidine -1-Oxyl (TPPA-TEMPO) on General Toxicity and Therapeutic
Efficiency of Cyclophosphamide on Transplantable Mouse Lymphosarcoma LS in Vitro and in Vivo

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MATERIALS AND METHODS

Animals

Male and female CBA mice (3 – 4 months of age) were obtained from the Experimental Animal Breeding Laboratory of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences. The animals were kept in plastic cages, 10-11 animals per cage, in a room with controlled temperature and lighting and free access to water and food (pelleted feed "Chara", Sergiyev Posad, Russia).

Experimental Tumor Model

The transplantable mouse tumor, **Lymphosarcoma LS**, was used in the experiments. Originally the tumor was induced in the laboratory of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences using nitrosomethyl urea in a CBA mouse and after a series of transplantations on syngeneic animals, it was transferred into an ascites form. It was maintained in the ascites form by weekly passages in the mice of this strain. When transplanted intramuscularly, tumor cells give rise to solid nodules demonstrating high sensitivity to alkylating agents; the use of relatively small doses of these drugs leads to a significant tumor regression [18, 19].

Cyclophosphamide (CP) used in experiments was produced by Biochimik, Saransk, Russia. It was administered into mice by parenteral means intraperitoneally (i.p.) or intravenously (i.v.) in a single- or multiple doses ranging from 20 to 100 mg/kg of body weight.

Mechlorethamine: (Nitrogen Mustard) was produced by Merck, Germany.

The Scheme of *in vivo* Therapeutic Experiment in Mice

Lymphosarcoma LS cells (2×10^6 in 0.1 ml of saline) were transplanted into 76 mice in the right thigh muscles. Immediately after transplantation, the mice were randomly divided into 7 groups. The schedule of experiment is presented in Table 1. The mice of one group (No.1) were used as an untreated control, whereas the mice from groups 2, 5 and 7 for 10 days were daily i.p injected with TPPA-TEMPO (30 mg/kg per injection). On the 10th day after the tumor transplantation the mice in groups 4, 5, 6 and 7 were treated i.p with CP (30 mg/kg), after which the mice from groups 3, 6 and 7 received 8 daily injections of TPPA-TEMPO as it had been done in the corresponding groups before starting CP therapy. From the moment of CP treatment until the death of the mice, tumor nodes were periodically measured with the calipers and the average volume of tumors per mouse in the group being calculated. The animals were observed until their death, considering their lifespan from the moment of the tumor transplantation, their body weight and the mass of the tumor.

Table 1: Schedule of the Therapeutic Experiment

Group	Treatment Before CP	CP	Treatment After CP
1	Control	-	-
2	TPPA-TEMPO 30 mg/kg i.p. daily from the 1st to the 10th day after tumor transplantation	-	-
3	-	-	TPPA-TEMPO 30 mg/kg i.p. daily from the 11th to the 17th day after tumor transplantation
4	-	CP in single i.p. injection at a dose 30 mg/kg on the 10th day after tumor transplantation	-

Table 1: Contd.,			
5	TPPA-TEMPO as in group 2	CP as in group 4	-
6	-	CP as in group 4	TPPA-TEMPO as in group 3
7	TPPA-TEMPO as in group 2	CP as in group 4	TPPA-TEMPO as in group 3

Evaluation of TPPA-TEMPO Effect on Tumor Growth after its Incubation with Tumor Cells Prior Transplantation

To find out whether TPPA-TEMPO has a direct cytotoxic effect on tumor cells or not, it was added at a dose of 10 mg/ml to their suspension immediately before inoculation into the mice or 0.5 hours before the implantation took place. In the latter case, in one group tumor cells were transplanted into the mice together with TPPA-TEMPO, whereas in another group the cells were washed from TPPA-TEMPO before the transplantation. In an additional group of mice, the tumor cells were implanted in the right femur, and TPPA-TEMPO was inoculated into the left one.

The Study of Possible Immunological Adjuvant Properties of TPPA-TEMPO

The experiments with the use of LS tumor cells have been carried out in CBA mice. For immunization, tumor cells (50×10^6 / ml of tissue culture) were incubated for 30 minutes with mechlorethamine ($10 \, \mu g/ml$) at 37° C. As it has been shown previously, this procedure does not lead to the formation of tumor from such cells, with their immunogenic properties being preserved. After washing, the tumor cells were suspended in physiological saline and the suspension was divided into 2 parts; 3 mg/ml of TPPA-TEMPO was added to one part and immediately after that, 0.1 ml of the suspension was inoculated into left thigh muscles of the mice. The mice of another group received by analogy tumor cells in physiological saline, whereas the third group of mice was not immunized. After 14 days, to test immunization effect, a freshly prepared suspension of LS tumor cells (4×10^5 per mouse) was inoculated into the muscles of the right thigh muscles of mice of all three groups. The animals were observed for 35 days, considering the number of the mice with arisen tumors and measuring the size of the latter.

In vitro **study of TPPA-TEMPO Activity**

Estimation of the Effect of TPPA-TEMPO on Tumor cells Viability

Suspension of tumor cells was taken from abdominal cavity of the mice on the 7th day after intraperitoneal transplantation of LS washed with Hanks solution twice and cultivated in 96-well plates in RPMI-1640 containing 10% of fetal calf serum, L- glutamine and gentamycin for 24 hours at $37°C$ in the atmosphere with 5% content of CO_2 and 85% humidity. TPPA-TEMPO was added before the beginning of cultivation in concentration ranging from 0.01 to 10 mg/ml. Proliferative activity was studied in MTT-test [20]. For this purpose, after incubation, 20 μl of MTT (3-[4,5-dimethyl-tiazol-2-il]-2,5-diphenyliphenyl tetrazolium) solution (5mg/ml) was introduced in each well. After the exposure within 3 hours liquid was removed and DMSO (200 mcl) was added; the obtained formasane salts were dissolved upon stirring at room temperature for 30 minutes. The development of coloring was registered by measuring optical density at wavelength 540 nm directly in the wells of the plate with LX multi scan.

Estimation of Direct Cytotoxicity of TPPA-TEMPO

LS cells were cultivated in 24-well plates in 1 ml of culture medium in concentration of

2×10^5 cells/well. The TPPA-TEMPO was introduced in experimental wells, and Hanks solution in control wells. One hour after the beginning of cultivation, the cells were re-suspended right in the wells, and a part of the suspension was taken in order to detect the number of living and dead cells. Estimation of cell viability was determined by trypan blue

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exclusion. For this purpose, a 5% solution of staining agent was added to the culture, and after 5 minutes the number of colored nonviable cells was microscopically detected and their percentage in culture was calculated. Each point was measured in triplicate.

Histological Examination of TPPO-TEMPO Toxicity

Mice were decapitated; the heart was excised and transferred on the ice immediately until the full cardiac arrest. Heart, lungs, liver, kidneys, spleen, and bone marrow were fixed in 10% neutral formalin for 24 h, and then embedded in paraffin. Paraffin sections (3 μm, Microm HM 430, Thermo Scientific) were stained by HE, PAS-reaction, and Giemsa staining.

Estimation of TPPA-TEMPO effect on Functional Activity of Macrophages

The functional activity of macrophages was estimated in a short-term culture with NBT (nitro blue tetrazolium)-test [21] in proper modification. Peritoneal cells of CBA mice were cultivated for 2 hours in RPMI medium containing L-glutamine, gentamicin and 10% fetal calf serum. Opsonized sheep erythrocytes or zymozan particles (Zymosan A, «Sigma»; 3 mg/ml) which were added on the formed macrophages cellular monolayer, were used as phagocytosis objects. The use of various phagocytosis objects allowed us to estimate the effect of the drug on phagocytosis mediated by different types of receptors. Cell activity was estimated with the use of spectrophotometric NBT (nitro blue tetrazolium)-test according to cellular reduction of indicator nitro blue tetrazolium by the cells. Macrophages were incubated for 1 hour with different concentrations of TPPA-TEMPO, and then their functional-metabolic activity was estimated.

The experimental data were subjected to statistical analysis carried out with "Statgrapfics 5.0" (Statistical Graphics Corp., the USA). The Student's *t*-test was chosen as a test of significance of the revealed intra-group variations. For variation validation the value of confidence probability $P < 0.05$ was considered sufficient.

RESULTS AND DISCUSSIONS

In vivo Investigations

Preliminary experiments in CBA mice have shown that a single i.p. administration of TPPA-TEMPO at the dose of 90 mg/kg causes 100% lethality, whereas at the dose of 70 mg/kg 5 of 6 mice died (83.3% lethality). Animals died within 10 – 30 minutes following lungs affection. In the dose of 50 mg/kg and lower, TPPA-TEMPO has not caused sudden deaths of animals, and after 10 daily administrations at the dose of 40 mg/kg TPPA-TEMPO has led to a certain decrease of body weight (less than 6%) and the death of 1 mouse of 8. On the basis of these data, in therapeutic experiments with chronic administration of TPPA-TEMPO we injected it in a single dose of 30 mg/kg.

In the therapeutic experiments an average volume of tumor transplants in the mice of 2, 5 and 7 groups after 10 injections of TPPA-TEMPO appeared to be reliably lower compared to the mice from 1, 3, 4 and 6 groups that did not receive the drug (1.3 ± 0.08 cm^3 and 1.8 ± 0.09 cm^3, accordingly, $p < 0.001$). Body masses (with tumor) of the mice that did not receive TPPA-TEMPO, increased by 10%, whereas body masses of those receiving the drug increased by only 4 % compared to the initial ones; after the deduction of tumor mass, the body mass accounted for approximately 4% and 0%, correspondingly. Consequently, a relatively long-term (daily for 10 days) administration of TPPA-TEMPO in a dose not causing the decline in natural body mass has resulted in an almost 30% inhibition of tumor growth, which testifies to a certain selectivity of its effect on the tumor. However, this effect has a slight impact on tumor growth rate after drug withdrawal (ref.: Figure 1, curves 1 and 2): all mice from group 2 which received TPPA-TEMPO and at a later date were

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left without the treatment died from tumor practically at the same time as the untreated control mice from group 1 (lifespan increased by only 14%, Table 1). Besides, administration of TPPA-TEMPO to the animals with advanced tumors (beginning with the 10th day after transplantation) has caused an insignificant decrease in the volume of the transplants (curve 3, Figure 1). The obtained results show that hardly can TPPA-TEMPO *per se* be treated as an independent antitumor drug, and the hopes to apply it in oncology can only be set on the combination with other antitumor compounds. Therefore, further we studied the efficacy of antitumor therapy with combined application of TPPA-TEMPO and CP. The results are shown in Figure 1 and Table 2.

Table 2: Lifespan and Tumor Mass in Mice with Solid Transplants of Lymphosarcoma LS Treated with CP Combined with Preliminary and/ or Subsequent Administration of TPPA-TEMPO (M±M)

Group Number	Number of Mice		Life span of Tumor Bearing Mice, Days	Mean Tumor Weight, G		Life Span of Mice Free of Tumor, Days
	All of Them	With Tumor		Per tumor Bearing Mice	Per Mouse	
1	11	11	17,0±1,2	3,9±0,58	3,9±0,58	-
2	10	10	19,4±1,8	4,3±0,58	4,3±0,58	-
3	11	11	16,4±2,0	3,1±0,26	3,1±0,26	-
4	11	11	27,9±3,5**	3,9±0,45	3,9±0,45	-
5	11	6	^{w}47,1±4,9**	4,0 ± 0,76	2,2±0,74	45; 60; >100; >100; >100
6	11	9	34,8±5,5**	2,6±0,50	^{v}2,1±0,52*	58; >100;
7	11	4	^{v}41.0±4.6***	3,6± 0,75	^{w}1,3±0,59**	16; 36; 37; 42; 48; 50; 66

Symbols (*, **, ***) indicate the values significantly different from those of untreated control (group 1), symbols ^{v} and ^{w} – significantly different from those of group 4, treated with cyclophosphamide (*p<0.05; **p<0.01; ***p<0.001; ^{v} p<0.05; ^{w} p<0.01).
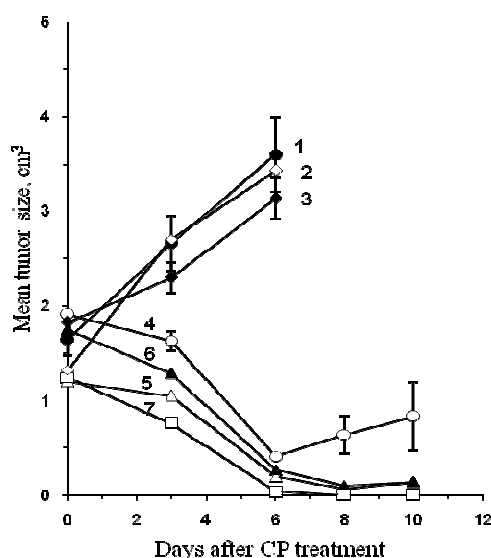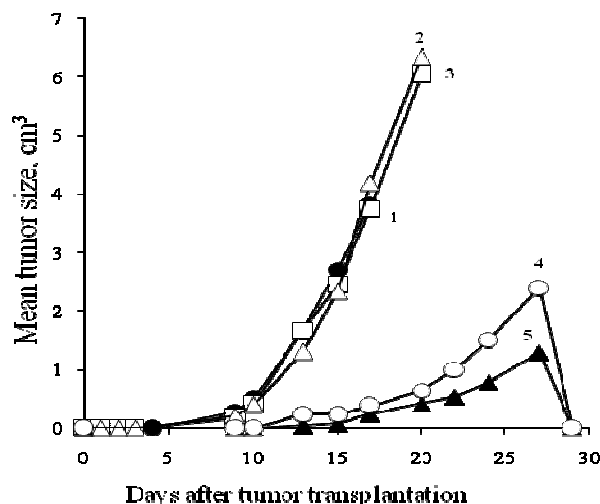

Figure 2

Figure 2 Influence of TPPA-TEMPO on the growth of intramuscular transplants of lymphosarcoma LS and on therapeutic effect of CP against the tumor under different drug administration schedules: 1 – control; 2 – administration of TPPA-TEMPO for the first 10 post-implantation days; 3 – administration of TPPA-TEMPO starting from the 11th to the

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17th days after tumor implantation; 4 – single dosing of CP on the 10th post-implantation day; 5 – administration of TPPA-TEMPO during the first 10 days after tumor implantation followed by a single dosing of CP; 6 – single dosing of CP on the 10th day after tumor implantation followed by an 8-times administration of TPPA-TEMPO; 7 – administration of TPPA-TEMPO for the first 10 days after tumor implantation, with a single CP dosing followed by an 8-fold injection of TPPA-TEMPO.

Figure 3 shows that after CP dosing, tumors tend to regress in the mice of all groups. Preliminary and posterior (after CP) administration of TPPA-TEMPO has led to a more expressed and lengthy effect. Thus, in the mice treated with only CP the tumor growth recommenced on the 8th day after the injection, but in the mice treated with CP in combination with TPPA-TEMPO the tumor volume continued decreasing. Table 2 shows that all mice treated with only CP (group 4) died from tumor on average 27.9 ± 3.5 days after its transplantation. In group 5, where CP was injected after TPPA-TEMPO, slightly more than a half of the mice (6 of 11) died from tumor at a later date, while 3 mice completely recovered from tumor and lived without tumor recurrence for more than 100 days. When TPPA-TEMPO was administered only after CP (group 6), 9 mice of 11 died from tumor and 2 mice completely recovered from tumor; only 1 mouse lived without tumor recurrence for 100 days. When TPPA-TEMPO was administered before and after CP (group 7), only 3 mice died with tumors, whereas the rest of the animals died at the same dates without tumors. Their body weight accounted for 80% of the initial, and three of them having paresis of hind limbs.

Thus, TPPA-TEMPO *per se* brings out a certain tumor growth inhibition, but in combination with CP it significantly increases the efficacy of antitumor therapy, which can be expressed in the increase of not only lifespan, but also the number of animals cured from tumor. However, the value of both effects appeared to be unequally dependent on the duration of TPPA-TEMPO application. Thus, 10-fold administration of TPPA-TEMPO before CP in group 5 has led to the recovery of less than a half of the mice (45.5%) but increased their average lifespan (more than 63 days) by 2.5 times, whereas 18-fold administration of TPPA-TEMPO (10 before and 8 after CP treatment) in group 7 has resulted in 63.6% animal recovery from tumor, with their lifespan (41 ± 4.6 days) being not more than 1.5 times longer compared to the mice treated with only CP (group 4). Consequently, when estimating the antitumor effect, the advantage of a lengthy application of TPPA-TEMPO was completely devalued in terms of mice lifespan estimation, which is highly likely to be caused by the side effects of TPPA-TEMPO on the organism of the animals.

In the sequencing experiments we studied the direct effect of TPPA-TEMPO on tumor cells. Figure 2 shows that tumors were growing slower than in the control mice when tumor cells were transplanted to the animals in the mixture with TPPA-TEMPO. Injection of TPPA-TEMPO immediately before the transplantation of tumor cells into the same place produced a similar effect on the tumor growth. However, when the tumor cells were treated with TPPA-TEMPO and 30 min later washed to remove nitroxide, or when TPPA-TEMPO was injected into contralateral limbs, the transplanted tumors were growing at the same rate as those in the control mice. Since TPPA-TEMPO inhibits tumor growth only when injected with tumor cells, it seems to interfere into the process of establishing relations between the tumor and the organism, presumably, strengthening the immune response of the latter.

Figure 3: TPPA-TEMPO Effect on the Growth of Intramuscular Transplants of Tumor LS
Depending on the Conditions of its Action on Transplanted Tumor Cells

To check this assumption we have immunized the mice with LS tumor cells pretreated with mechlorethamine, injecting them in the left thigh both in saline (group 2) and in TPPA-TEMPO solution (group 3). The mice of group 1 were left without immunization. Antineoplastic alkylating agent mechlorethamine in appropriate concentration is known to inactivate tumor cells, making them capable of causing a specific immune response but preventing the initiation of tumor growth. After a month, all mice were inoculated in the right thigh with 1×10^6 of viable LS cells. The measurements of the formed tumor size have shown (Figure 3) that immunization by tumor cells in saline (group 2) markedly suppresses the tumor growth, whereas immunization by them in mixture with TPPA-TEMPO significantly decreases the effect of immunization. The obtained results show that tumor growth inhibition with TPPA-TEMPO does not provide the evidence that it is caused by stimulation of a specific immune response on the tumor, rather it seems to be a result of some other effects of TPPA-TEMPO.
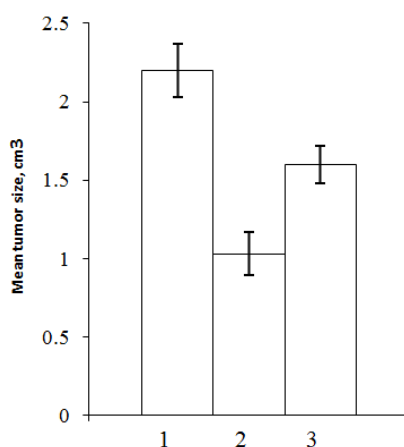

Figure 4: Effect of TPPA-TEMPO on the Immunogenicity of LS Cells.1-Control, 2-
Immunization with LS cells, 3- Immunization with LS Cells + TPPA-TEMPO

In order to clarify whether TPPA-TEMPO activity is associated with its general antioxidant/prooxidant properties of the nitroxide group, we compared it with another cell-permeable nitroxide with similar redox characteristics, the Tempol. The tumor cells were transplanted into mice in the mixture containing 1 mg of TPPA-TEMPO, equimolar (0.3 mg)

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and equitoxic (2.7 mg per mouse) quantity of Tempol. After 14 days, the mean volume of the tumors in control mice transplanted with tumor cells in saline was 5.0 ± 0.51 cm^3; transplantation of tumor cells with TPPA-TEMPO gives rise the mean tumor volume of 3.3 ± 0.28 cm^3 ($p < 0.05$), whereas transplantation with Tempol – 4.6 ± 0.24 cm^3 and 4.9 ± 0.32 cm^3 in large and small doses correspondingly. Consequently, TPPA-TEMPO ability to inhibit the growth of lymphosarcoma LS is apparently associated with the specific accumulation in mitochondria or with individual characteristics of the molecule.

To determine the cytotoxic effect of TPPA-TEMPO we conducted a histological examination of the internal organs of mice in groups 2 and 3. In the mice of group 2 morphological changes revealed predominantly in heart, lungs, liver, and kidneys. In all organs we observed fresh hemorrhages, marked hyperemia of vessels, and generally intramural arteries spasm. TPPA-TEMPO cytotoxic effect on cardiomyocytes manifested in the appearance of small foci of dystrophically altered cells, mostly lytic changes in varying degrees (Figure 4), which affected the myofibrillar apparatus. In lungs we registered focal atelectasis, probably, as a result of bronchial obstruction in blood. Alveolar septa were irregularly thickened because of edema, hemorrhage and mononuclear infiltration (Figure 5); besides, there was a moderate peribronchial and perivascular edema. Vessels of various calibers were irregularly hyperemic. In kidneys we observed the irregular expansion of Bowman's space and the collapse of vascular bundles (Fig. 6). The epithelium of the proximal tubule was edematous, often with the lumen obturation. In liver we noted the irregular distribution of glycogen granules in hepatocytes and hepatic lobules; several hepatic lobules had lots of PAS-positive inclusions. Sinusoids and central veins were dilated and irregularly hyperemic. In spleen and bone marrow we observed no significant changes.
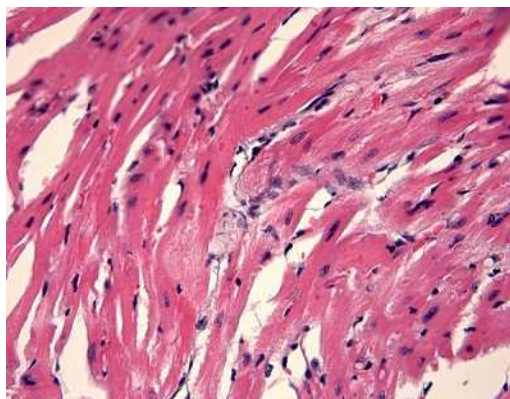
**Figure 5: Small Foci of Cardiomyocytes Lytic Changes in the
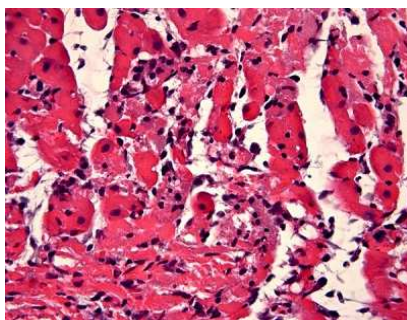Heart of 2 Group Mice. H&E Staining, x400**
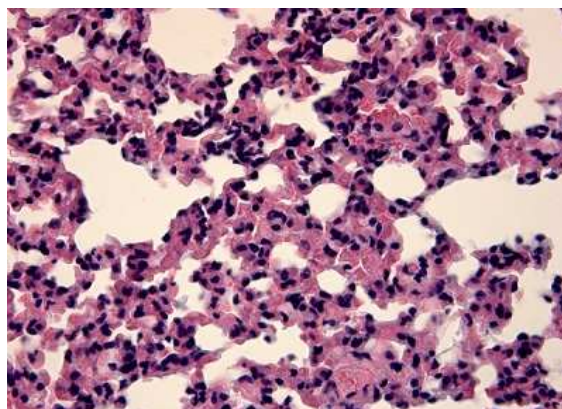
**Figure 6: Focal Atelectasis and Diffuse Mononuclear Infiltration of Alveolar
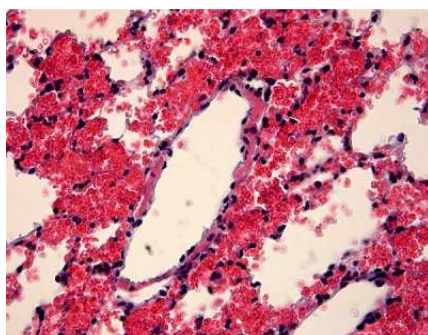Septa in the Lungs of 2 Group Mice. H&E Staining, x400**
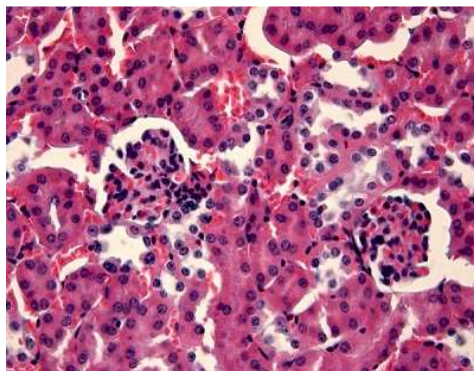
**Figure 7: The Expansion of Bowman's Space in the Kidneys of 2 Group Mice. The Epithelium of the
Proximal Tubule is Edematous. Vessels are Hyperemic. H&E Staining, x400**
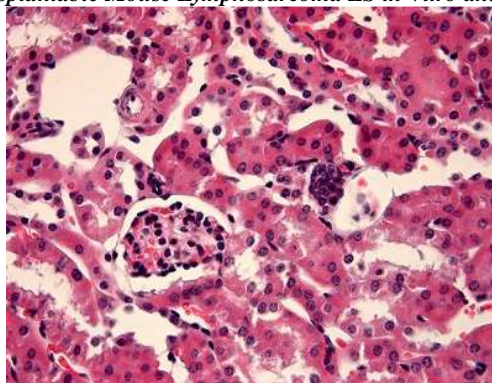
In the mice of group 3 structural changes in heart, lungs, liver, and kidneys were more pronounced. In heart lytic changes of cardiomyocytes were more significant compared with group 2, but the damages remained predominantly focal. Because of these changes, in the myocardium developed small focal mononuclear infiltration, which consisted mostly of macrophages (Figure 7). In lungs we recorded subtotal atelectasis, alveolar walls were edematous, blood soaked with its release into alveoli (Figure 8). Therefore, we registered a moderate bronchospasm, and a moderate diffuse mononuclear infiltration of alveolar septa and the formation of small peribronchial and perivascular infiltrates. In kidneys we recorded a significant glomerular atrophy as a result of its degradation. Bowman's spaces were profoundly expanded, and some glomeruli were collapsed (Figure 9). Epithelium of proximal tubules exposed to destructive changes, often looked edematous. Tubular lumens were filled with flaky substance or were collapsed. In liver we observed a focal degeneration of hepatocytes, a decrease of PAS-positive inclusions, and a pyknosis of nuclei in separate hepatocytes. In spleen and bone marrow we observed no significant changes.

**Figure 8: Focal Lytic Changes of Cardiomyocytes and Mononuclear
Infiltration in the Heart of 3 Group Mice. H&E Staining, x400**

**Figure 9: Subtotal atelectasis and mononuclear infiltration in the lungs of 3 group mice.
The lumens of alveoli are filled with blood. H&E staining, x400.**

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Figure 10: Atrophy and Destruction Of separate Glomeruli, the Expansion of Bowman's Space, and Diffuse Mononuclear Infiltration in the Kidneys of 3 Group Mice. The Epithelium of the Proximal Tubule is Edematous. Tubular Epitheliocytes are Dystrophic and Destructively Changed. H&E Staining, X400

In Vitro Investigations

To elucidate the results obtained in the experiments on mice we studied the effect of TPPA-TEMPO on some experimental models *in vitro*.

The study of cytotoxic activity of TPPA-TEMPO was carried out in the culture of LS cells. It has been revealed that at concentration of 10 mg/ml corresponding to that we used when 30 min incubated it with tumor cells prior to transplantation them into mice (Figure 3), TPPA-TEMPO after 1 hour exposition showed insignificant toxic effect on tumor cells (12.8 % dead cells against 10.4 % dead cells in control). Naturally, that one to two order smaller doses of TPPA-TEMPO showed insignificant influence on cell viability after 24 hours incubation (Table 3).

Table 3: Viability of Ls Cells (10^6 Cells/Ml) after 24 Hours Incubation with Tppa-Teмпo

Concentration of TPPA-TEMPO(mg/ml)	Number of dead cells x 10^5 (%)	
	Experiment I	**Experiment II**
0 (Control)	0.54 ± 0.06 (12.9 %)	0.18 ± 0.023 (8.5 %)
0,01	0.50±0.06 (14.3 %)	0.13 ± 0.009 (10.2 %)
0,1	0.43±0.19 (11.4 %)	0.16 ± 0.033 (11.0 %)
1,0	0,77±0,17 (18.0 %)	0.19± 0.019 (12.6 %)

However, co-cultivation/incubation of LS cells with TPPA-TEMPO in the dose of 0.1-1 mg/ml for 24 hours practically dose-dependently inhibits their proliferating activity (Table 4). Since no significant cytotoxic effect of TPPA-TEMPO on tumor cells was observed, we assume that the mechanism of the nitroxide effect on tumor growth *in vivo* implies in inhibition of tumor cells proliferation or stimulation of their phagocytosis by macrophages.

To check the last assumption, a comparative study has been carried out of the effect TPPA-TEMPO produces on phagocytosis of opsonized sheep erythrocytes and zymosan particles. The increase of Fc-receptors (FcRs) expression is known to be an indicator of macrophages' activation. The decreased expression of mannoze-fucose receptors (MFRs) seems to be another specific change of membrane phenotype accompanying the activation of macrophages. In both cases, the dependence of TPPA-TEMPO effects on its concentration has been studied.

Table 4: Proliferation Rate of LS Cells (10^6 cells/ml) after 24 Hours
Incubation with Different Concentration of TPPA-TEMPO

Concentration of TPPA-TEMPO (mg/ml)	The Level of Reduced MTT (Optical Units)
0 (Control)	2.36 ± 0.056 (100 %)
0,01	1.90 ± 0.096** (80.5 %)
0,1	2.02 ± 0.140* (85.6 %)
1,0	1.94 ± 0.081** (82.2%)

Note: In brackets, survival index of cells against control rate is given. (*) indicates significant differences with control group (physiological saline):* $p<0.05$; **$p<0$.

It has been shown that TPPA-TEMPO produces a stimulating effect on FcR-direct phagocytosis in concentrations ranging from 0.01 to 0,1 mg/ml (Table 5). It is necessary to note that concentration-dependence of the effect has not been observed. Presumably, at higher concentrations TPPA-TEMPO inhibits functional activity of macrophages, similar to those producing onto lymphosarcoma LS cells.

Table 5: The Effect of TPPA-TEMPO on Functional Activity of Peritoneal
Macrophages in ICR Mice *in Vitro* Against Opsonized Sheep Erythrocytes

Concentration of TPPA-TEMPO (mg/ml)	The level of reduced of NST (optical units)
0 (control)	0,265±0,005
0,01	0,322±0,005*
0,1	0,304±0,006*
1,0	0,273±0,005

*- the differences are significant with respect to the control group ($p< 0, 001$)

`Inversely, redox activity of macrophages was inhibited in conditions of zymosan-induced NBT-test. It has been recorded a more than twofold decrease in the indicators of NBT-test, characterizing oxigen-dependent potential of macrophages cultivated for 60 minutes in different concentrations of TPPA-TEMPO, which denotes the suppression of "respiratory explosion" of macrophages (Table 6).

Table 6: The Effect of TPPA-TEMPO on Functional Activity of
Peritoneal Macrophages *in Vitro* Against Zymosan

Experiment Number	Concentration of TPPA-TEMPO (mg/ml)	The Level of Reduced of NST (Optical Units)	
		Experiment I	Experiment II
I	0 (Control)	0,585±0,013 (100 %)	0,285±0,006 (100 %)
	0,01	0,470±0,008* (80.3 %)	0,245±0,005* (86.0 %)
	0,1	0,386±0,005* (66.0 %)	0,198±0,006*(69.5 %)
	1,0	0,220±0,006* (37.6 %)	0,127±0,006* (44.6 %)

*- the differences are valid with respect to control group ($p< 0, 001$)

Thus, the described above *in vitro* studies in the tumor cells cultures clearly showed that small doses of TPPA-TEMPO (0,01-1,0 mg/ml) suppress the tumor cells metabolism and their proliferation, without affecting cell viability.

CONCLUSIONS

TPPA-TEMPO shows a complex pattern of antitumor activity. On the one hand, it is poorly active *per se*, but increases antitumor activity of CP against lymphosarcoma LS.

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TPPA-TEMPO tends to inhibit the growth of lymphosarcoma LS apparently irrespective of its radical and antioxidant properties but rather due to the preferential localization in mitochondrias. The possibility exists that TPPA-TEMPO in doses used activates macrophages by provoking the effect similar to oxidative stress. On the other hand, it can disturb the mitochondrial functions intercepting carriers of electrons, i.e., along with activation of ROS formation, TPPA-TEMPO may protect the cells from their harmful influence. Both of these possibilities may be differently manifested in the tumor cells and in the cells participating in tumor resistance.

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